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For the development of HER2/neu mimicking anti-idiotype antibodies (Ab2s), groups of female BALB/c mice were immunized with three sets of monoclonal antibodies (Ab1), designated 520C9, 741F8 and 454C11, directed at distinct antigenic determinants of HER2/neu. Hybridomas were generated from each treatment group and one, stable Ab2 producing hybrid each of 520C9 and 741F8 were obtained, and cloned twice by limiting dilution. The isotypes of 520C9 and 741F8 Ab2's were IgG1k by ELISA. 520C9 and 741F8 Ab2 cells were used to produce mouse ascites and the Ab2 purified by affinity chromatography and confirmed by SDS-PAGE. A competitive binding assay using Ab2, Ab1 and the human breast cancer cell line SK-BR-3 (which express HER2/neu on their cell surface), showed that 50µl of each of 520C9 and 741F8 Ab2 containing supernatants inhibited the binding of Ab1 to SK-BR-3 cells by ~80%.The specificity binding study showed that the binding of 741F8 Ab2 was to those antibodies directed against HER/2neu. A competitive binding assay using 741F8 Ab2, Ab1 and SK-BR-3 cells showed that 10ng of purified 741F8 Ab2 inhibited the binding of Ab1 to SK-BR-3 cells by ~50%. Polyclonal anti-anti-idiotype antibodies (Ab3), were generated in rabbits using either purified 520C9 Ab2 or 741F8 Ab2. The Ab3 reacted with HER2/neu positive tumor cells suggesting the presence of anti-tumor (Ab1′) antibodies.

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Malaya Chatterjee 7/28/97

PI - Signature Date

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INTRODUCTION

Breast cancer is a major cause of cancer deaths in women. The incidence of breast cancer has steadily increased over the last two decades and patients with recurrent disease are not curable by standard therapies. In human breast cancer, amplification and overexpression of the cell membrane protein HER2/neu, which is not present on normal breast tissue, has been observed to occur in a significant number of tumors. In 189 primary human breast cancers, HER2/neu was found to be amplified from 2 to over 20 fold in 30% of the tumors (1). Patients with multiple copies of the HER2/neu gene in DNA from their tumors had a shorter time to relapse as well as a shorter overall survival (1-3), indicating that HER2/neu gene amplification was prognostic for both disease behavior and clinical outcome in these patients. Not only were increased copy numbers of HER2/neu in breast cancers related to a poorer prognosis, but gene amplification of HER2/neu correlated with lymph node involvement (1-5), histological grade (5,6), negative estrogen receptor content (7,8), early recurrence (4,7), increased mitotic activity (9), all of which are considered to be poor prognostic indicators. In a retrospective study, the expression of HER2/neu determined immunohistochemically in positive breast cancer samples from 253 patients found that HER2/neu positive breast cancers behaved more aggressively in the first 2-3 years following diagnosis (10). Several studies have shown that overexpression of HER2/neu occurs in as many as 15-40% of breast cancers and that overexpression of HER2/neu is associated with poor survival (1-3). Therefore HER2/neu present on the surface of overexpressing breast cancer cells offers a good target for immunotherapy. It has been shown that monoclonal antibodies raised against the human protein that bind to the extracellular domain of HER2/neu can inhibit the growth of tumor cells *in vitro* (11-13) and *in vivo* (14). A recent report has determined that breast cancer specific cytotoxic T lymphocytes recognize a 9 amino acid peptide from the transmembrane portion of the HER2/neu protein (15).

Since cancer patients are often immunosuppressed and also tolerant to some tumor-associated antigens such as HER2/neu, triggering an active immune response to such antigens represents a challenge in cancer therapy. One approach has been to use tumor derived material as the immunogen. As an alternative to the use of tumor antigens or tumor cells, the network hypothesis of Neils Jerne (16) offers a different approach to vaccine therapy using the so-called internal image antigens (17-21). According to the network concept, immunization with a given antigen will generate the production of antibodies against this antigen termed Ab1. This Ab1 can generate a series of anti-idiotype antibodies termed Ab2. Some of these Ab2 molecules can effectively mimic the three dimensional structures of external antigens. These particular anti-idiotypes called Ab2ß, which fit into the paratopes of Ab1, can induce specific immune responses similar to the nominal antigen. Anti-idiotype antibodies of the ß type express the internal image of the antigen recognized by the Ab1 antibody and can be used as surrogate antigens. Immunization with Ab2β can lead to the generation of anti-anti-idiotype antibodies (Ab3) that recognize the corresponding original antigen. Indeed, human trials using anti-idiotype monoclonal antibodies (Ab2β) to stimulate immunity against the patients own tumor has shown promising results. Objective clinical improvement has been observed in patients with colorectal cancer, melanoma and T-cell lymphoma (for reviews see 22,23).

Our overall aim has been to apply this approach to the treatment of breast cancer by attempting to develop monoclonal anti-idiotype antibodies against HER2/neu starting

with a series of mouse monoclonal antibodies (Ab1) that recognize different epitopes of HER2/neu (24-26). We selected 3 monoclonal antibodies 520C9, 741F8 and 454C11 which recognize distinct antigenic determinants on HER2/neu. These monoclonal antibodies appear highly selective; immunoperoxidase staining of normal human tissues showed negligible staining with these antibodies (26). Furthermore, when conjugated to ricin A, these antibodies produced immunotoxins selectively cytotoxic to SK-BR-3 breast cancer cells (26). The restricted specificity of these monoclonal antibodies together with their high binding capacity to a representative breast carcinoma cell line SK-BR-3, make them excellent target for generating Ab2 hybridomas. The use of various Ab2 that mimic multiple epitopes on the tumor cell surface may increase immunotherapeutic efficacy of Ab2 immunizations. Furthermore, the internal image antigens (Ab2) generated against these 3 monoclonal antibodies may cover almost all of breast tumors expressing HER2/neu and in many cases will complement each other. Our overall goal is to develop monoclonal anti-idiotype antibodies as a surrogate for the tumor associated antigen HER2/neu to be used for the treatment of breast cancer.

BODY

Materials and Methods

Animals

Female BALB/c mice, 6-8 weeks old and male New Zealand rabbits 4-6 months old from Harlan Laboratories.

Cells

Established human cell lines were obtained from the American Type Culture Collection (Rockville, MD). SK-BR-3 cells were cultured in McCoy's Medium and HBL 100 cells cultured in RPMI 1640 each supplemented with 10% fetal calf serum.

Antibody

Mouse monoclonal antibodies 520C9, 741F8 and 454C11 were obtained from Chiron Corp. These antibodies were used to immunize female BALB/c mice for the production of anti-idiotype antibodies. Ascites of Ab2 hybridomas was prepared by injecting pristane-primed BALB/c mice with $2x10^6$ to $1x10^7$ viable cells into the peritoneal cavity. The IgG fraction was isolated from ascites using a Hi-Trap Protein G affinity column (Pharmacia, Piscataway, NJ). The purity of the isolated IgG was assessed by SDS-PAGE (10% mini-gel) (27-29).

Immunization of BALB/c mice

Groups of BALB/c mice, 6-8 weeks old were immunized with monoclonal antibodies 520C9, 741F8 or 454C11 (Ab1's). The first injection was administered i.p. with 100µg of Ab1 mixed with Freund's complete adjuvant. The second injection was administered s.c. two weeks later with 100µg of Ab1 in Freund's incomplete adjuvant. Subsequent injections were given i.p. at two week intervals, with 100µg of Ab1 coupled to KLH and mixed with Freund's incomplete adjuvant. Mice were bled two weeks following each injection and the sera checked for anti-idiotype activity by Sandwich RIA using the respective Ab1. Mouse IgG1 was used as control. Three days prior to the fusion, mice were boosted with Ab1 (100µg in PBS) injected intravenously into the tail vein.

Coupling of antibody with keyhole limpet hemocyanin (KLH)

Antibody stock solution (1mg/ml) was mixed with KLH (1mg/ml) in PBS in the presence of glutaraldehyde (0.05%). The mixture was rotated for 2 hours at room temperature and then dialyzed for 24 hours against PBS in the cold (27).

Fusion

Fusion of BALB/c mouse spleen cells with non-secretory P3-653 myeloma cells using 50% polyethyleneglycol (3,400 mwt. Aldrich Chemical Co., Milwaukee, WI) was carried out as previously described (27-29). Hybrids were selected using hypoxanthine-aminopterine-thymidine media.

Selection of anti-idiotype antibodies (Ab2)

Initial screening of hybridomas for anti-idiotype antibody production was done by Sandwich RIA using the respective Ab1 at a concentration of 250 ng/well to coat plates (27,28). After overnight incubation at 4^0C, plates were blocked with 1% bovine serum albumin in PBS for 30 minutes. Thereafter, 50 µl of undiluted hybridoma culture supernatants were incubated for 2 hours at room temperature with continuous shaking. After washing with PBS, plates were further incubated for 2 hours at room temperature with ~50,000 cpm of I-125-radiolabelled Ab1. Stable Ab2 producing fusion wells were then further checked for reactivity toward an unrelated mouse monoclonal Ab1, mouse IgG1 and KLH by Sandwich RIA as described above. Ab2 producing fusion wells were expanded in hypoxanthine-thymidine media and cloned twice by limited dilution to obtain a single population of Ab2 producing cells using monolayers of mouse peritoneal macrophages as feeder cells (27,28).

Isotype determination

The Ig isotype of the cloned Ab2 producing hybridomas was determined by ELISA. Essentially, ELISA plates were coated with goat-anti-mouse immunoglobulins (250ng/well) that specifically bind mouse immunoglobulins (Cappel, Westchester, PA). After overnight incubation at 4^0C, plates were blocked with 2% bovine serum albumin in PBS containing 0.05% Tween 20 for 2 hours. Thereafter, 50µl of undiluted hybridoma culture supernatant were added in triplicate and incubated for 2 hours at room temperature with gentle shaking. After washing with PBS containing 0.05%Tween 20, plates were incubated for 2 hours with specific alkaline phosphatase labeled anti-mouse immunoglobulins (against IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, kappa chain, lambda chain). Plates were washed and p-nitrophenolphosphate in diethanolamine buffer added to each well (50µl of 1mg/ml) and the color developed read in an ELISA reader at 10, 20 and 60 minutes at 405nm (28,29).

Immunization of animals with anti-idiotype antibodies (Ab2)

Once Ab2 were identified they were injected into BALB/c mice as previously described (27,28). Male New Zealand rabbits were injected s.c. with 500µg of purified Ab2 in Freund's complete adjuvant on day 0 and the same amount of Ab2 in Freund's incomplete adjuvant 2, 4 and 6 weeks following the first immunization (27,28). Animals were bled 2 weeks following the third and fourth immunizations and the sera checked for anti-Ab2 activity by Sandwich RIA using plates coated with purified Ab2 (250ng/well).

Cell binding assays

(1). Radioimmunoassay (RIA)

This assay was performed in disposable microfold 96-well microfilter plates. The plate was first treated with 10% fetal calf serum and 1% bovine serum albumin in PBS. Then, 50µl or 100µl aliquots of Ab2 culture supernatants were added to individual wells containing $5x10^5$ viable SK-BR-3 cells (in 50µl PBS). Ab1 (~50,000 cpm in 50µl PBS) was then added to each well and the plate shaken for 2 hours at room temperature. After incubation, the plate was washed 3 times with PBS containing 10% bovine serum albumin with suction. The radioactivity in the washed filter paper was measured in a gamma counter (Packard Instruments).

Percent inhibition of the assay was calculated according to the formula:

$$\%\text{Inhibition}=1-(Rt)/(Rmax) \times 100$$

where Rt was the average cpm of the experimental well with Ab2 and Rmax was the average maximum binding in the absence of inhibitor.

(2). Immune flow cytometry

Binding of Ab3 to tumor cell lines was also determined by immune flow cytometry. Antigen positive SK-BR-3 cells ($1x10^6$/well) were reacted with Ab1 and Ab3 in a volume of 100µl at 4^0C for 1 hour. After washing, the cells were incubated with either goat-anti-mouse F(ab')2 IgG-FITC labeled antibody or goat-anti-rabbit IgG-FITC labeled antibody (Tago Inc. Burlingame, CA) for 30 minutes at 4^0C. The cells were washed twice, fixed in 2% paraformaldehyde and analyzed by immune flow cytometry (FACS STAR, Becton Dickinson). Antigen negative HBL 100 human breast cells were used as a control in this assay.

Results

Generation of monoclonal anti-idiotype hybridomas

The Ab1's 520C9, 741F8 and 454C11 were used to generate Ab2 in BALB/c mice. Seven immunizations with 520C9, five immunizations with 741F8 and three immunizations with 454C11 were required to generate a sufficient Ab2 response in BALB/c mice for fusions. That is, a 1:160 dilution of immune sera produced more than double that of pre-immune sera in binding to the respective Ab1 by Sandwich RIA. Furthermore, immune sera exhibited no binding to KLH or mouse IgG. Culture supernatants of primary fusion cells were initially screened on the basis of their binding to the respective Ab1 and their failure to bind to mouse IgG1, KLH or another unrelated mouse monoclonal Ab1 (mAb 8019). Only one stable Ab2 producing hybridoma was obtained for each of 520C9 and 741F8. These Ab2 hybridomas were cloned twice by limited dilution. No Ab2 producing hybridomas were obtained for 454C11.

A preliminary study was conducted to determine whether 520C9 Ab2 and 741F8 Ab2 would compete for the binding of a fixed amount of Ab1 (~100,000 cpm) to SK-BR-3 cells, which express the tumor antigen HER2/neu (Figure 1). As little as 50µl of culture supernatant inhibited the binding of radiolabeled Ab1 to SK-BR-3 cells by ~80%.

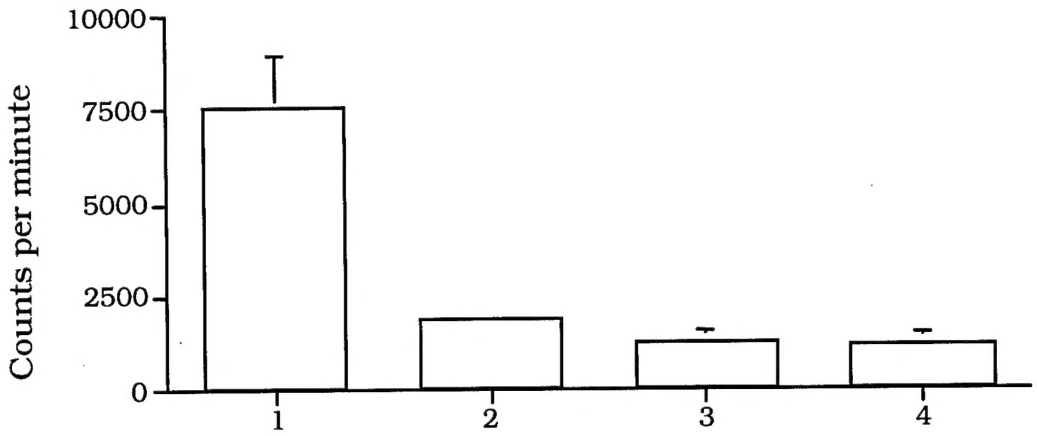


Figure 1. Inhibition of 520C9 and 741F8 binding to SK-BR-3 cells by Ab2 containing hybridoma supernatants.

Viable SK-BR-3 cells (2-3x10^6) were plated in disposable microfold 96 well chamber microfilter plates and reacted with different amounts of Ab2 containing culture supernatants and a fixed amount of I-125 Ab1 (100,000 cpm). Values are the mean + SE of 3 determinations. 1= SK-BR-3 cells + I-125-Ab1 (520C9 or 741F8). 2= SK-BR-3 cells + I-125-Ab1 + mouse Immune sera (1/20 dilution). 3 and 4= SK-BR-3 cells + I-125-Ab1 + hybridoma supernatant (50µl and 100µl respectively) of 520C9 Ab2 or 741F8 Ab2.

Purification of Ab2

520C9 and 741F8 Ab2 producing hybridomas were used to produce mouse ascites (a rich source of Ab2), and the Ab2 purified by Hi-Trap Protein G affinity chromatography (Figure 2). SDS-PAGE (10% mini-gel) showed 2 bands of approximate molecular weight 25,000 and 50,000 which correspond to the molecular weights of mouse IgG light and heavy chains respectively.

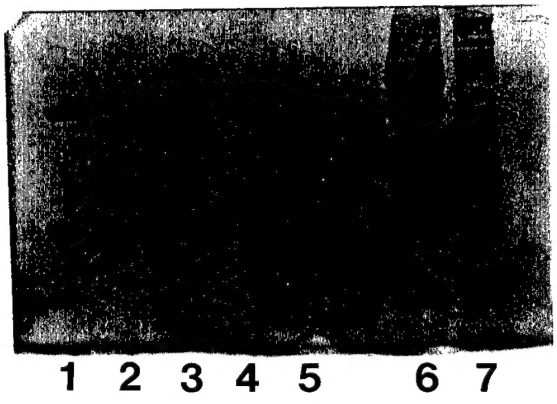


Figure 2. SDS-PAGE of 520C9 and 741F8 Ab2's purified from mouse ascites using a Hi-Trap Protein G column.

Lanes 1, 2, 3 = Column eluents containing ~5,2,1µg purified protein 520C9 Ab2. Lanes 4, 5 = column eluents containing ~ 5, 2µg purified 741F8Ab2 and Lane 6=Mouse IgG (2µg, Sigma Chemical Co. St Louis MO). Lane 7= Molecular weight markers (10µl, BioRad Labs., Hercules CA).

Isotype determination

The IgG isotypes of 520C9 and 741F8 Ab2's were determined by ELISA to be IgG1k.

Binding specificity of 520C9 and 741F8 Ab2's.

To determine whether the anti-520C9 antibodies were specific for the Ab1 and not directed against allotypic or isotypic determinants, we set up a Sandwich RIA in which plates were coated with 250ng of mouse IgG1 or an unrelated mouse monoclonal antibody (8019, IgG1k), and then incubated them with 50µl of Ab2 hybridoma supernatant. The binding of Ab2 to the proteins on the plate was measured using radiolabeled Ab1. Less than 300 cpm bound to plates coated with mouse IgG1 or mAb 8019 and reacted with Ab2 hybridoma supernatant, whereas 1,500-4,000 cpm bound to 520C9 coated plates reacted with Ab2 hybridoma supernatant.

A more comprehensive specificity study carried out with 741F8 Ab2 showed a significant cross reaction with the other two monoclonal antibodies directed against HER/2neu, that is 454C11 and 520C9 which recognize closely related epitopes on HER/2neu to 741F8. Minor cross reaction was seen with the remaining monoclonal antibodies, the most being with MC10, a monoclonal antibody directed against human milk fat globule membrane antigen (HMFG) (Figure 3).

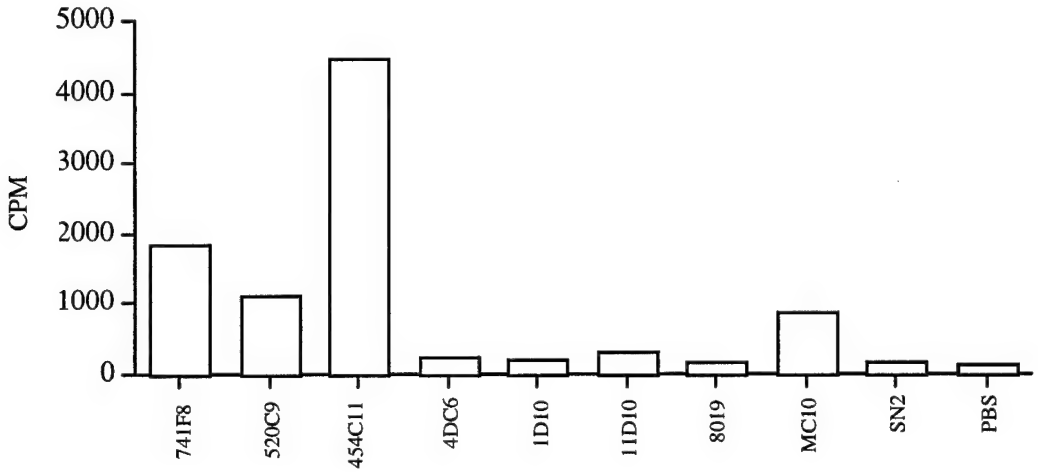


Figure 3. Anti-idiotype specificities of 741F8 (IgG1k). Binding of 741F8 Ab2 to various mouse monoclonal antibodies was determined by indirect RIA. The isotypes of the monoclonal proteins used to coat the plates were 741F8 (IgG1k), 520C9 (IgG1k), 454C11 (IgG2a), 4DC6 (IgG1λ), 1D10 (IgG1k), 11D10 (IgG1k), 8019 (IgG1k), MC10 (IgG2a) and SN2 (IgG1k). The results are presented as mean cpm of 3 separate wells. The SD of the data was less than 10% for the assay.

An inhibition study was performed to determine whether purified 741F8 Ab2 would compete for the binding of a fixed amount of Ab1 to SK-BR-3 cells, which express the tumor antigen HER/2neu. Prior to this, experiments to determine SK-BR-3 cell number and the amount of radiolabeled 741F8 Ab1 to use were conducted to select optimal conditions for the competitive binding assay using purified 741F8 Ab2. Binding of 741F8

Ab1 was linear with respect to cell number and $3x10^5$ cells and $2x10^5$ cpm of 741F8 Ab1 were selected for the competitive binding study (Figure 4).

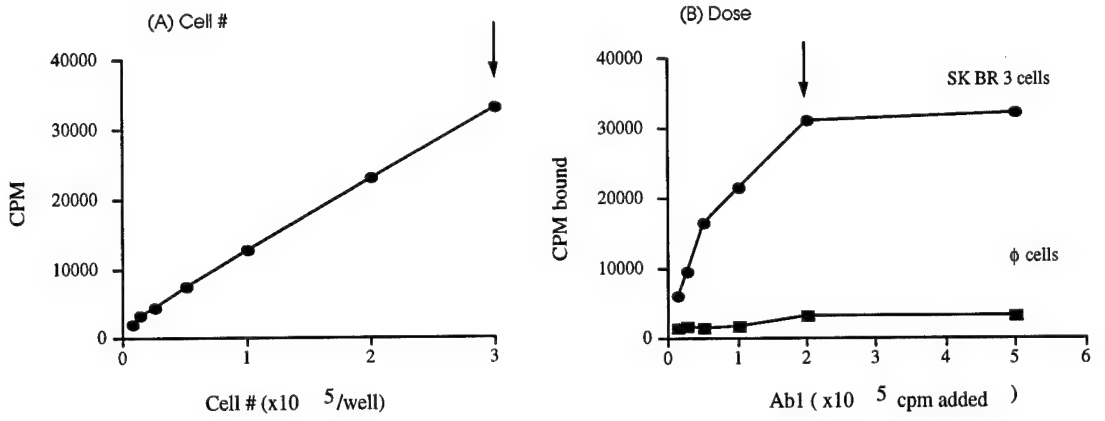


Figure 4.Binding of 741F8 Ab1 to SK-BR-3 cells. (A) Effect of cell number. Different numbers of SK-BR-3 cells were incubated with a fixed amount of radiolabeled 741F8 ($2x10^5$cpm) for 2 h at room temperature with shaking, washed and the filter counted in a gamma counter. (B) Effect of 741F8 Ab1 concentration. Different amounts of radiolabeled 741F8 Ab1 were incubated with $3x10^5$ SK-BR-3 cells for 2h at room temperature with shaking, washed and the filters counted in a gamma counter. The results are presented as the mean cpm of 3 separate wells. The SD of the assay was less than 10% for the assay.

From the inhibition study it appears that as little as ~10ng of 741F8 Ab2 inhibited the binding of radiolabeled 741F8 Ab1 to SK-BR-3 cells by ~50% (Figure 5). No binding was observed with the control human breast cell line, HBL-100, which does not express the tumor associated antigen HER/2neu on its surface.

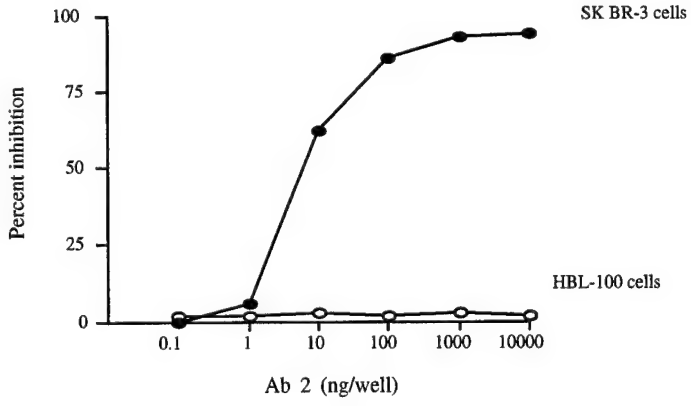


Figure 5. Inhibition of 741F8 Ab1 binding to SK-BR-3 cells by purified 741F8 Ab2. Different concentrations of 741F8 Ab2 and a fixed amount of radiolabeled 741F8 Ab1 were added to each well and incubated for 2h at room temperature with shaking with $3x10^5$ SK-BR-3 cells (antigen positive) or HBL-100 cells (control cells).Plates were then washed to remove unbound Ab1 and the filters counted in a gamma counter. Results are the mean percent inhibition of 3 separate wells. The SD was less than 10% for the assay.

Generation of polyclonal Ab3

If the Ab2's are true internal images, then they should induce the production of antigen specific Ab3 in the absence of exposure to antigen in a genetically unrestricted way and across species barriers (22, 23). To determine this mice and rabbits were immunized with either 520C9 Ab2 or 741F8 Ab2 for the production of Ab3's that might share idiotopes with Ab1 and exhibit identical binding specificity.

Comparison of the binding of Ab1 and Ab3 mice or rabbit sera to tumor antigen positive cells.

To ascertain whether these Ab3 are directed against the tumor associated antigen HER2/neu, the binding of mice or rabbit Ab3 sera to human SK-BR-3 cells which express the antigen and to a control human breast cell line HBL 100 which do not express this antigen was determined by immunofluorescence (Figure 6).

(A) 520 C9

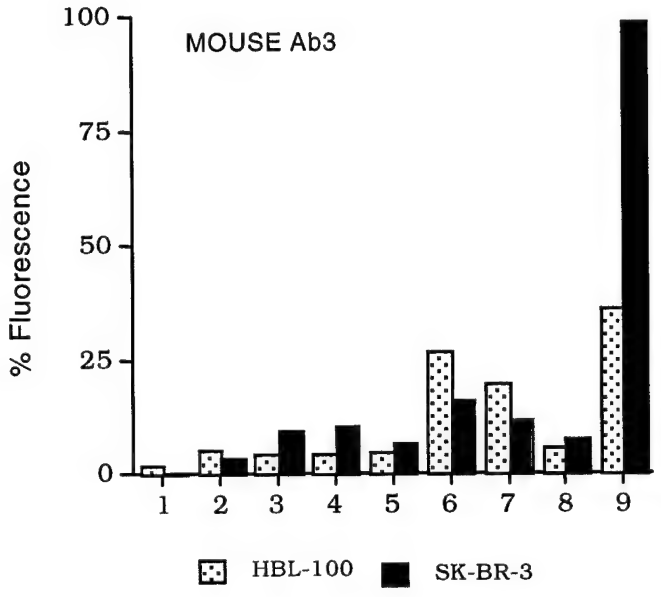
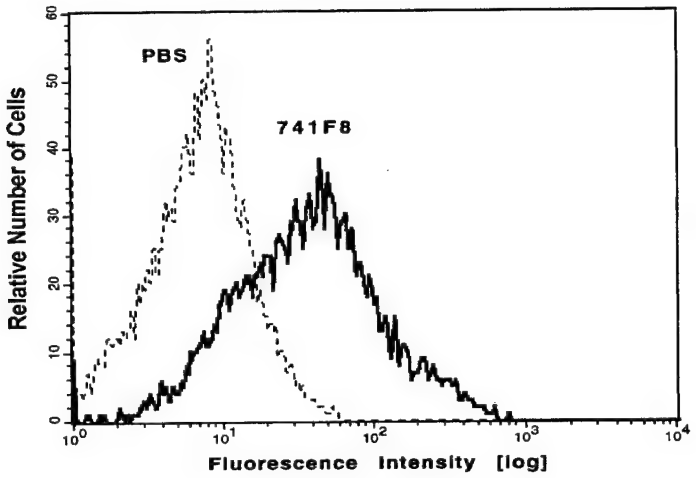


Figure 6. Binding of Ab3 sera from mice immunized with 520C9 Ab2 and Ab3 sera from rabbits immunized with 741F8 Ab2 with SK-BR-3 cells determined by immunofluorescence.
(A): 1= Cells only. 2= Cells + second antibody (anti-mouse FITC). 3,4,5= Cells + pre-immune sera (1/50, 1/100 and 1/500 dilution respectively). 6,7,8= Cells + immune sera (1/50, 1/100 and 1/500 dilution respectively). 9= Positive control consisting of cells + mouse Ab1 (5µg).

(B) 741 F8

i.



ii.

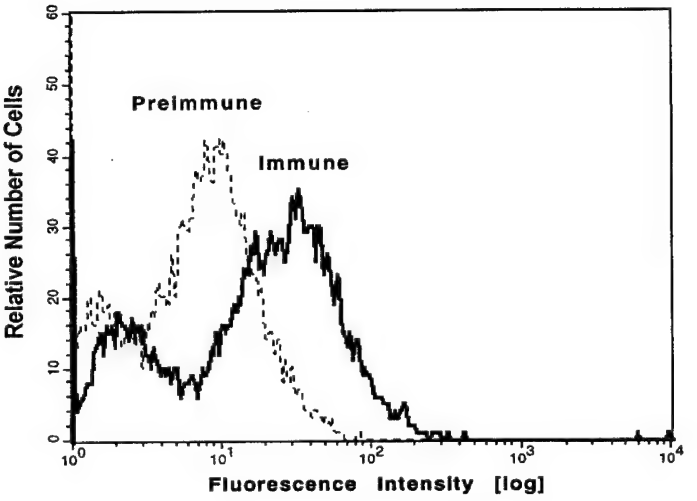


Figure 6 (B): SK-BR-3 cells were treated in panel (i) with PBS (---) or Ab1 741F8, 5 µg (—) and in panel (ii) with pre-immune sera, 1/50 dil (---) or 741F8 Ab2 immune sera, 1/50 dil (—) and the Fluoresence intensity was determined by FACS analysis.

CONCLUSIONS

The Ab1's 520C9, 454C11 and 741F8 were used to generate Ab2's in female BALB/c mice. Despite a high fusion frequency of over 80%, only one stable Ab2 producing hybridoma was obtained for each of 520C9 and 741F8 which were then cloned twice by limited dilution to obtain a single population of Ab2 producing cells. No Ab2 producing hybridomas were obtained for 454C11 Ab1.

A preliminary study was conducted to determine whether 520C9 and 741F8 Ab2's in hybridoma culture supernatants exhibited high competitive binding with their respective Ab1's to SK-BR-3 cells; a breast cancer cell line which expresses the antigen HER2/neu to a high degree on its cell surface. We found that 50µl of each of the Ab2 containing culture supernatants inhibited the binding of Ab1 to SK-BR-3 cells by approximately 80%. Furthermore, the specificity of 520C9-Ab2 and 741F8Ab2 binding (determined by Sandwich RIA), was directed only to their respective Ab1's as no binding was observed to mouse IgG, KLH or to other unrelated mouse monoclonal antibodies we have in our laboratory (mAb8019/11D10).

The specificity of 741F8 Ab2 binding was directed to its respective Ab1 and to the other two monoclonal Ab1's also directed against HER/2neu. Very little binding was observed with 741F8 Ab2 to the other mouse monoclonal Ab1's tested except for MC10, which is also a mouse monoclonal Ab1 directed against HMFG present in malignant breast tissue. This suggests that 741F8 Ab2 contains a public idiotope which is shared among various anti-HER/2 neu and anti-breast cancer antigen antibodies. This anti-Id could be a potential reagent for treating a large number of breast cancer patients positive for the above antigens.

Studies were conducted to determine whether purified 741F8 Ab2 exhibited high competitive binding with its respective Ab1 to SK-BR-3 cells, a breast cancer cell line which expresses the antigen HER2/neu to a high degree on its cell surface. Experiments to determine optimal conditions for determining percent inhibition of Ab1 binding to SK-BR-3 cells by Ab2 were completed i.e. the number of cells and the amount of Ab1 to add was established. We found that Ab1 binding to SK-BR-3 cells was linear with respect to cell number over the cell numbers examined. We selected a cell number of $3x10^5$ cells. We then determined the amount of radiolabeled Ab1 to use by incubating these cells with different amounts of radiolabeled 741F8 Ab1. Amounts of $2x10^5$ cpm Ab1 and above appeared to be "saturating" and so we chose to use $2x10^5$ cpm for the inhibition experiments. Using $3x10^5$ cells/well and $2x10^5$ cpm of 741F8 Ab1 we found that ~10ng of purified 741F8 Ab2 inhibited the binding of Ab1 to SK-BR-3 cells by approximately 50%.

Using purified 520C9 Ab2 and 741F8 Ab2 we determined whether these Ab2's were able to induce the production of antigen specific Ab3 in mice and rabbits. Both mice and rabbits showed significant immunity i.e. Ab3 response (assessed by the binding of sera to plates coated with Ab2 in a Sandwich assay). 520C9 Ab3 mice sera showed moderate binding whereas, 741F8-Ab3 rabbit sera showed significant binding to Her/2neu positive SK-BR-3 cells in immunofluoresence assay. There was negligible binding to control HBL-100 cells. The results suggest that 520C9 Ab2 and 741F8 Ab2 might be useful for the induction of tumor-specific immunity in breast cancer patients.

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Bibliography

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